

## PHYLOGENETIC STUDIES OF SOME SPECIES OF THE GENUS *Coffea*. II - NUMERICAL ANALYSIS OF ISOENZYMATIC DATA

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### ABSTRACT

Thirteen species of *Coffea* were studied for five enzymatic systems, including alpha and beta esterase, alkaline phosphatase, acid phosphatase, malate dehydrogenase and acid dehydrogenase. Three coefficients of similarity: Simple Matching, Jaccard and Ochiai and three different clustering methods: Single Linkage, Complete Linkage and Unweighted Pair Group, using Arithmetic Averages (UPGMA) were used to analyse the data.

The phylogenetic relationships among the twelve diploid species and between them and the tetraploid species *C. arabica* showed that similarity among species of the same subsection is not always greater than among species of different subsections. In addition, although there are several similarity groups in common, established by isoenzymatic polymorphism, morphological characteristics, chemical data, crossability and geographic distribution, there is no common trend among the phylogenetic relationships as indicated by all these different evaluating procedures.

### INTRODUCTION

The broad distribution of the species of the genus *Coffea* in Africa, as well as the difficulties involved with maintaining a comprehensive live collection in one given environment, are two of the major problems hindering a good understanding of the phylogenetic and taxonomic relationships among them.

The most complete taxonomic classification of this genus, based on morphological and ecological data, was provided by Chevalier (1947). However, with

the exploration of new areas, in Africa many new species have been described, mainly by Leroy (1980, 1982) and Bridson (1982). Data about the ecological conditions under which the species are found and about the genetic relationship of some species have also been collected (Carvalho and Monaco, 1967; Charrier, 1975, 1978; Carvalho *et al.*, 1985). These new data have not corroborated the distribution of species within sections and subsections proposed by Chevalier (1947). According to Charrier (1975), the genus *Coffea* includes only the sections *Eucoffea* and *Mascarocoffea*, while the sections *Paracoffea* and *Argocoffea* (Chevalier, 1947) form the new genera *Paracoffea* and *Argocoffea*, respectively. Wrigley (1988), presented the classification proposed by Leroy (1980), in which three genera belonging to the tribe Coffeae are recognized: genus *Coffea* L. composed of the subgenera *Coffea*, *Psilanthopsis* (Chev.) Leroy and *Baracoffea* (Leroy) Leroy; genus *Psilanthus* Hook. f. composed of subgenus *Afrocoffea* (Moens) Bridson, and genus *Nostolachma* T. Durand. The genus *Coffea*, subgenus *Coffea* contains by far the most species and includes all those commercially used, however, as is said by Wrigley (1988), breeders and growers still tend to adopt Chevalier's classification, although it is rather poorly defined by morphological characters. The subgenus *Psilanthopsis*, genus *Coffea*, based on one species, is doubtfully worth recognition in Bridson's opinion (1982). The subgenus *Baracoffea* was based on the Madagascar species *C. grevei* Drake ex Chev. and Leroy (1982) also included in it, two other Madagascar species and one species from Kenya and Somalia. According to Wrigley (1988) this taxon is certainly worth recognition and is interesting from an evolutionary point of view as it seems to form a link between *Coffea* and *Psilanthus*. The existence of the monotypic genus *Psilanthopsis* (Chevalier, 1947) was also contested by Carvalho and Monaco (1967) and Carvalho *et al.* (1985), among several other propositions. Furthermore, these authors and Charrier (1975) reported high crossability among species of different subsections and sections, with a large production of hybrids.

The use of isoenzymes to investigate genetic problems has been demonstrated in other plant species (Brown and Allard, 1970; Allard *et al.*, 1971; Hamrick *et al.*, 1979; Hamrick and Loveless, 1986; Buckley *et al.*, 1988). In the present research, phylogenetic and taxonomic relationships among thirteen species of the genus *Coffea* were evaluated by using isoenzymes as genetic markers and the data were analyzed through numerical taxonomy.

## MATERIALS AND METHODS

The species studied belong to the genus *Coffea*, according to the classification of Chevalier (1947), with modifications introduced by Carvalho and Monaco (1967), and Carvalho *et al.* (1985). The species *C. mauritiana* and *C. bengalensis* belong to the sections *Mascarocoffea* and *Paracoffea*, respectively. The other eleven species which

include *C. arabica*, *C. canephora*, *C. congensis*, *C. eugenioides*, *C. liberica*, *C. dewevrei*, *C. brevipes*, *C. stenophylla*, *C. racemosa*, *C. kapakata* and *C. salvatrix*, belong to different subsections of the section *Eucoffea*. All the material was obtained from the Coffee Germplasm Bank of the Department of Genetics of the Instituto Agronômico de Campinas, State of São Paulo, Brazil. Carvalho *et al.* (1985) have enumerated all possible origins of these 13 species. The *C. congensis* plants from the Coffee Germplasm Bank are not typical of *C. congensis* and there is a strong possibility that they are a hybrid between *C. canephora* cv Robusta and *C. congensis*, known by the name of Congusta. Only later, real representatives of the species *C. congensis* were introduced in the Coffee Germplasm Bank. *C. mauritiana* which was introduced in the Campinas collection through Cenargen (Centro Nacional de Pesquisas de Recursos Genéticos e Biotecnologia - EMBRAPA), is also not typical when compared to the description provided by Chevalier (1947). The available samples of this species have smaller leaves than the originals, thus presenting unexpected morphological similarities with *C. brevipes* (Carvalho, personal communication).

Electrophoresis to separate isoenzymes was performed at 4°C and 60 mA on a starch gel. For the study of alkaline phosphatases, acid phosphatases and esterases, the starch gels were prepared at 15% in the buffers 0.2 M Tris-citrate pH 8.3 and 0.2 M lithium borate pH 8.3 (9:1, respectively), according to Scandalios and Sorenson (1977). For the study of malate dehydrogenases and acid dehydrogenases, starch gels at 15% prepared in the buffer 0.2 M Tris-citrate pH 7.0, in a proportion of 1 part of the buffer to 60 parts of water, according to Scandalios and Sorenson (1977) were used.

To separate isoenzymes from acid phosphatase, alkaline phosphatase and esterase, a 0.2 M Tris-citrate pH 8.3 electrode buffer was used, and to separate isoenzymes from malate dehydrogenase and acid dehydrogenase a 0.1 M Tris-citrate pH 7.0 electrode buffer (Scandalios and Sorenson, 1977) was used. The procedures were considered complete when the front reached 9 cm from the origin.

After electrophoresis was completed the gels were sliced horizontally into 3 layers about 0.3 cm thick. All gels were incubated in the appropriate staining solutions (Steiner and Joslyn, 1979) at 37°C in the dark for different time periods, which depended upon the enzymatic system in question. The gels were then rinsed in distilled water and fixed in a solution of ethanol: acetic acid: water (5:5:1, respectively) for 8 hours. The relative migration ( $R_m$ ) of each isoenzyme in each enzymatic system was calculated by dividing the migration distance of each band in centimeters by the migration of the front.

The species were referred to as operational taxonomic units (OTUs), according to a list of characters (presence or absence of each enzymatic activity region or band). To determine the similarity among the OTUs, three coefficients of association were used: "Simple-Matching (SM), the Jaccard (J), and the Ochiai (O)", according to Sneath and Sokal (1973). The strategy used to accomplish the analysis was sequential,

agglomerative, hierarchical and not superimposed. Methods for clustering, unweighted pair group arithmetic averages (UPGMA), complete linkage (CL) and single linkage (SL) were utilized (Sneath and Sokal, 1973; Rohlf, 1988).

## RESULTS

The general isoenzymatic patterns obtained from each species for each analyzed enzymatic system are shown in Figures 1 and 2.

The results obtained from the Simple-Matching coefficient of similarity submitted to the 3 clustering methods, the Single Linkage, Complete Linkage and the UPGMA using all isozymes detected in the five different enzymatic systems studied are shown, respectively, in Figures 3, 4 and 5.

The results obtained from the Jaccard's coefficient of association are shown in Figures 6, 7 and 8, grouped respectively by Single Linkage, Complete Linkage and UPGMA methods. Figures 9, 10 and 11 refer to the results obtained from the use of the Ochiai's coefficient of association, grouped by Single Linkage, Complete Linkage and UPGMA methods, respectively.

## DISCUSSION

In all clusters obtained from the different coefficients of association utilized, six species formed three distinct pairs, sharing greatest similarity. One pair was composed of *C. canephora* and *C. congensis*, species which belong to the same subsection, share great morphologic similarity (Chevalier, 1947) and have a high degree of crossability (Carvalho *et al.*, 1985). Only with the flavonoid studies (Lopes and Shepherd, 1991), these two species showed a lower level of similarity.

*C. salvatrix* and *C. eugenioides* seem to be closely related and formed the second pair. These species belong to two different subsections, present distinct morphological characteristics (Chevalier, 1947) and have shown a low degree of crossability (Carvalho *et al.*, 1985). However, upon closer examination of the alkaline phosphatase, alpha and beta esterase isoenzymatic patterns, it can be seen that these two species share some specific bands, mainly those of the cathodic group. A similar observation was found with the flavonoid studies (Lopes and Monaco, 1979, Lopes and Shepherd, 1991) where these species also shared specific compounds. Isoenzymatic and flavonoid studies seem to indicate that there was introgression of *C. salvatrix* into *C. eugenioides*. Since morphological variability more than likely evolves through a selective process and electrophoretic variability is thought to be primarily neutral (Turner *et al.*, 1979), the level of morphological differentiation probably increased between these two species because of geographical isolation. However, some isoenzymatic alleles remained in common.

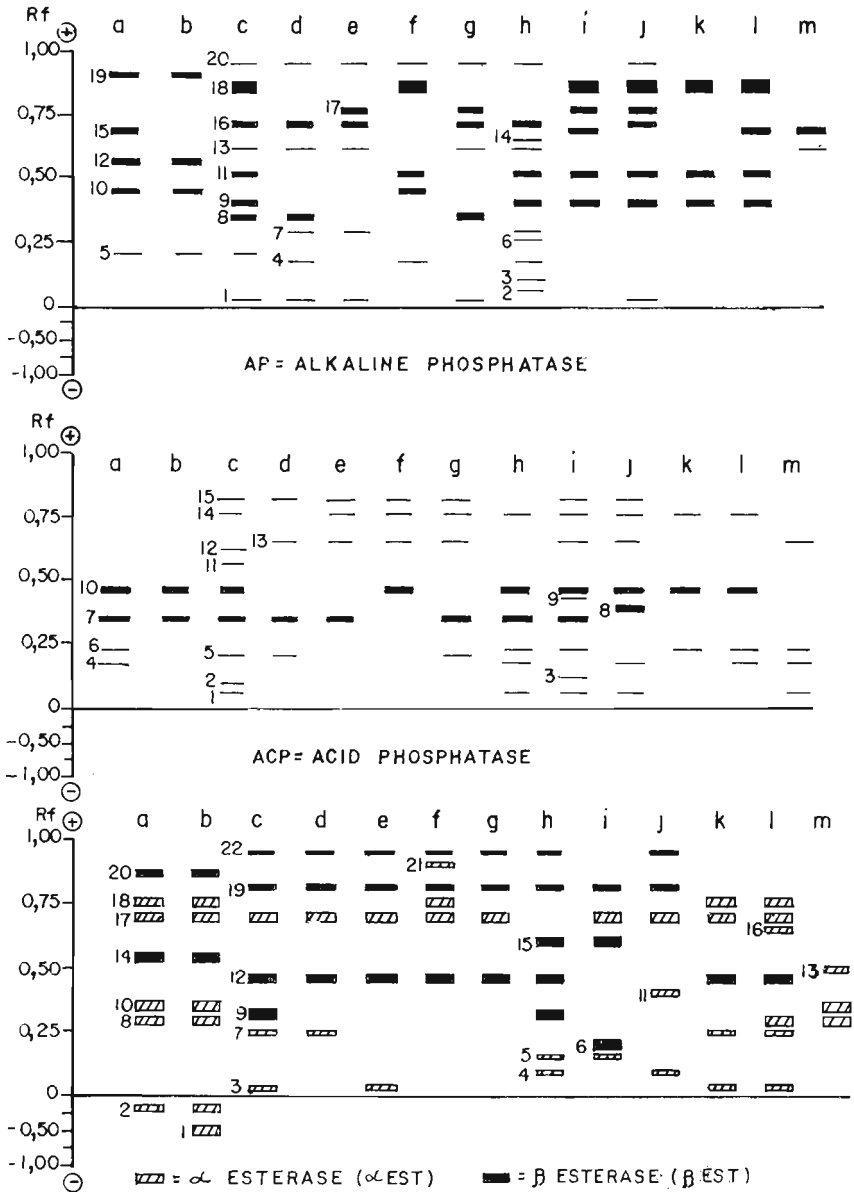


Figure 1 - Isozyme phenotypes observed in thirteen species of coffee (genus *Coffea*) to AP, ACP and EST enzymatic systems. a. *C. eugenioides*; b. *C. salvatrix*; c. *C. dewevrei*; d. *C. liberica*; e. *C. canephora*; f. *C. arabica*; g. *C. congensis*; h. *C. stenophylla*; i. *C. racemosa*; j. *C. kapakata*; k. *C. brevipes*; l. *C. mauritiana*; m. *C. bengalensis*.

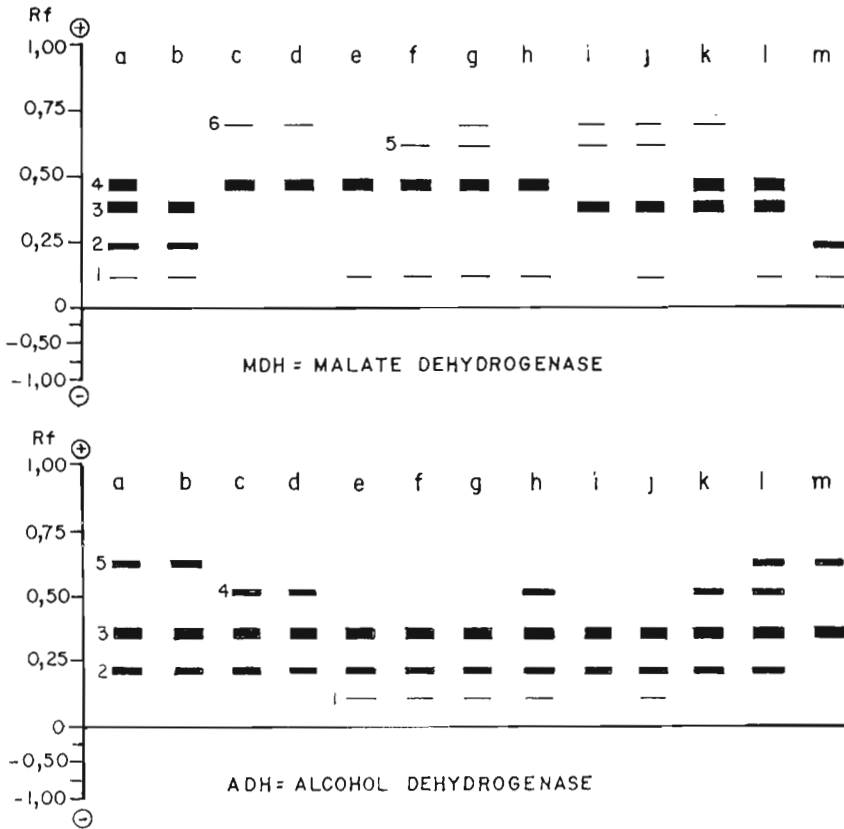


Figure 2 - Isozyme phenotypes observed in thirteen species of coffee (genus *Coffea*) using MDH and ADH enzymatic systems. a. *C. eugenioides*; b. *C. salvatrix*; c. *C. dewevrei*; d. *C. liberica*; e. *C. canephora*; f. *C. arabica*; g. *C. congensis*; h. *C. stenophylla*; i. *C. racemosa*; j. *C. kapakata*; k. *C. brevipes*; l. *C. mauritiana*; m. *C. bengalensis*.

The third pair observed in this study was composed of *C. mauritiana* and *C. brevipes*. This pair presented a high degree of similarity, which was never observed using morphological data (Chevalier, 1947) or level of crossability (Carvalho *et al.*, 1985). Of course, different forces must act on morphological and electrophoretic variants. The relative importance of these factors vary according to the presumed overall neutrality of isoenzyme variants and to the strong directional selection for particular morphological

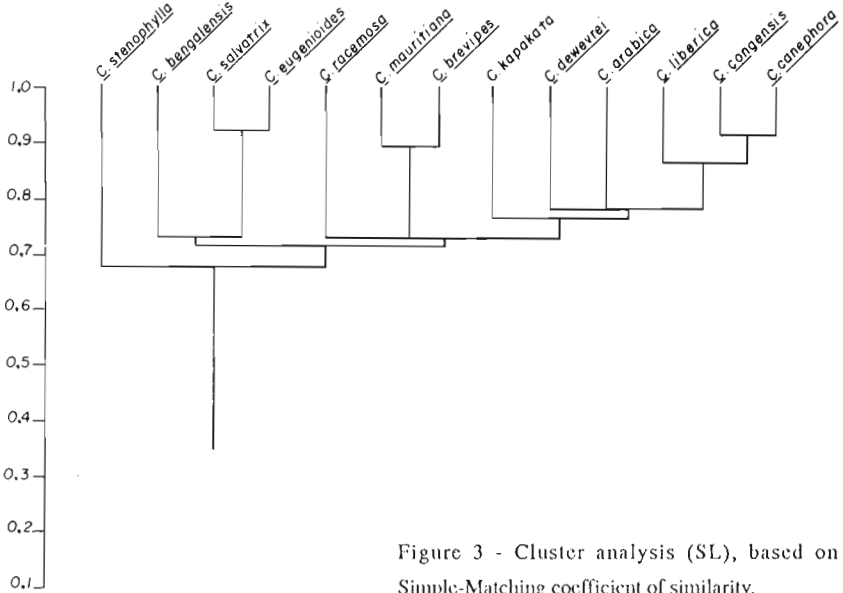


Figure 3 - Cluster analysis (SL), based on the Simple-Matching coefficient of similarity.

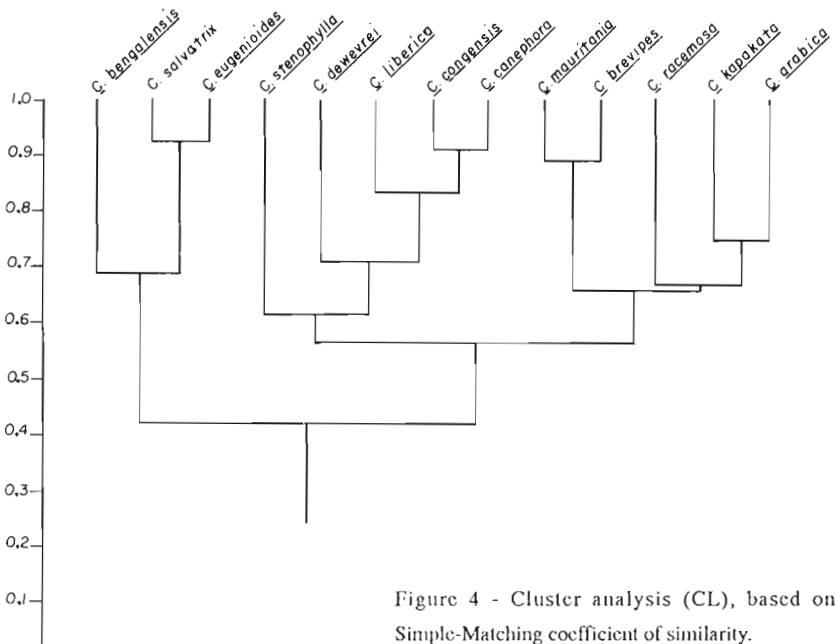


Figure 4 - Cluster analysis (CL), based on the Simple-Matching coefficient of similarity.



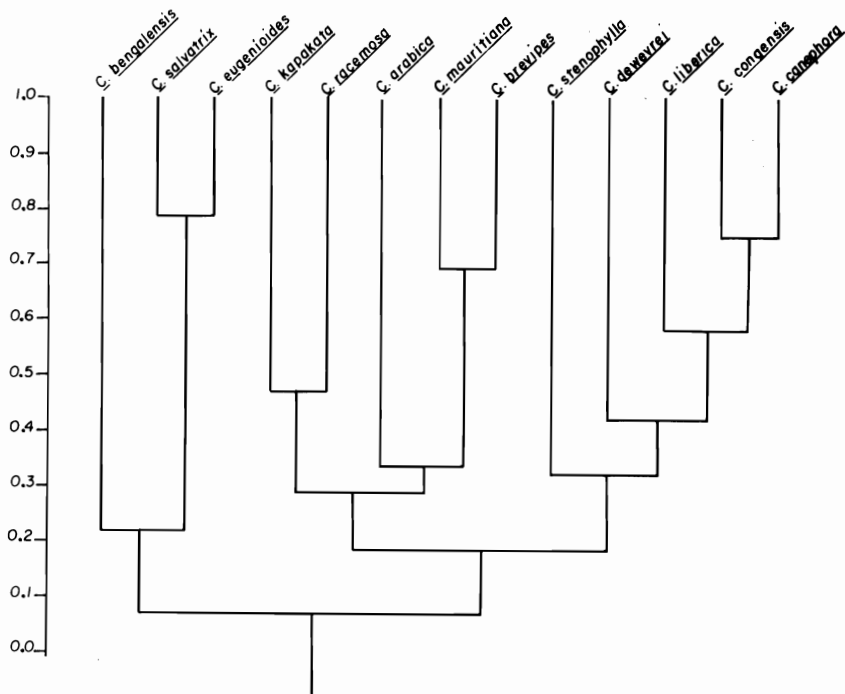


Figure 7 - Cluster analysis (CL), based on the Jaccard coefficient of similarity.

characteristics (Wolff, 1991). However, it is impossible to infer anything else since the *C. mauritiana* samples analysed share abnormal similarities with *C. brevipes* and these individuals could conceivably not be true representatives of *C. mauritiana*.

The high degree of affinity observed between *C. dewevrei* and *C. liberica* using morphological studies (Chevalier, 1942, 1947) was not confirmed in the present study where, at the electrophoretic level, a much higher degree of differentiation existed between these two species. In this case, these species are similar to each other, but they are close at the same level to other species. This also was observed in the crossability data (Carvalho *et al.*, 1985). In spite of the assumption presented by Chevalier (1942, 1947) that a common ancestor is shared by *C. liberica* and *C. dewevrei*, it is possible that the morphological similarity observed between them is a consequence of adaptation to the geographic areas in which they occur.

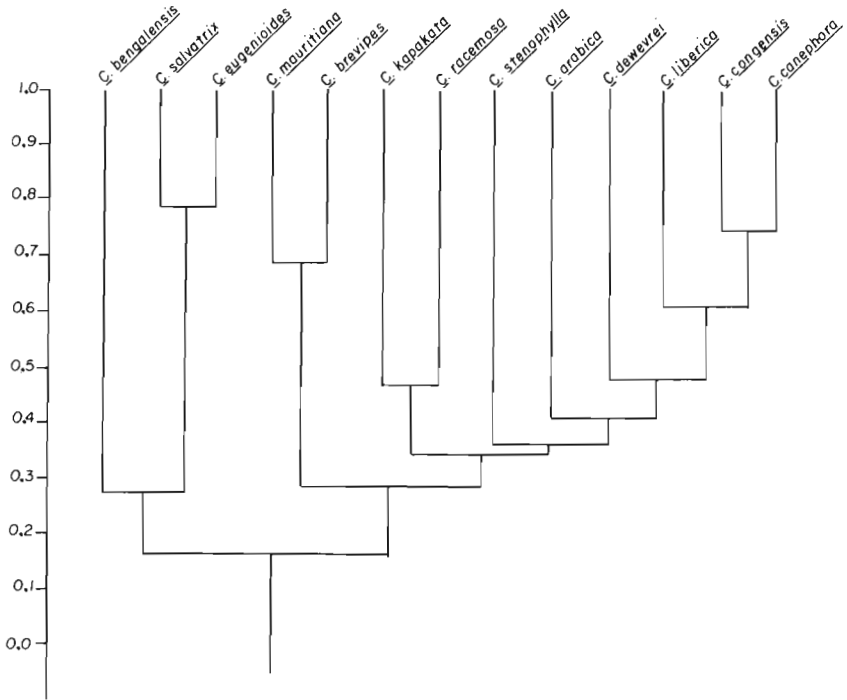


Figure 8 - Cluster analysis (UPGMA), based on the Jaccard coefficient of similarity.

The degree of affinity observed between *C. kapakata* and all other species studied in this research seems to justify its inclusion in the genus *Coffea*, as was previously proposed by Carvalho and Monaco (1967). This species, in a significant number of the clusters obtained, was closer to *C. arabica* than to the other species. In fact, Carvalho *et al.* (1985) obtained a high level of crossability between *C. kapakata* and the other species of the subsection *Erythrocoffea* (*C. congensis*, *C. canephora* and *C. eugenioides* and *C. arabica*), the best one reached by *C. arabica*.

In the majority of clusters, *C. arabica*, the only tetraploid species of the genus *Coffea*, an allotetraploid, does not show affinity with any of its putative parent species *C. canephora* and *C. congensis*. Even though it is close to these species, it never forms a special group of similarity with them and it also presents the same level of proximity with *C. dewevrei* and *C. kapakata*. In this study *C. arabica* presents a low degree of affinity to *C. eugenioides* in relation to the enzymatic systems studied. However, the

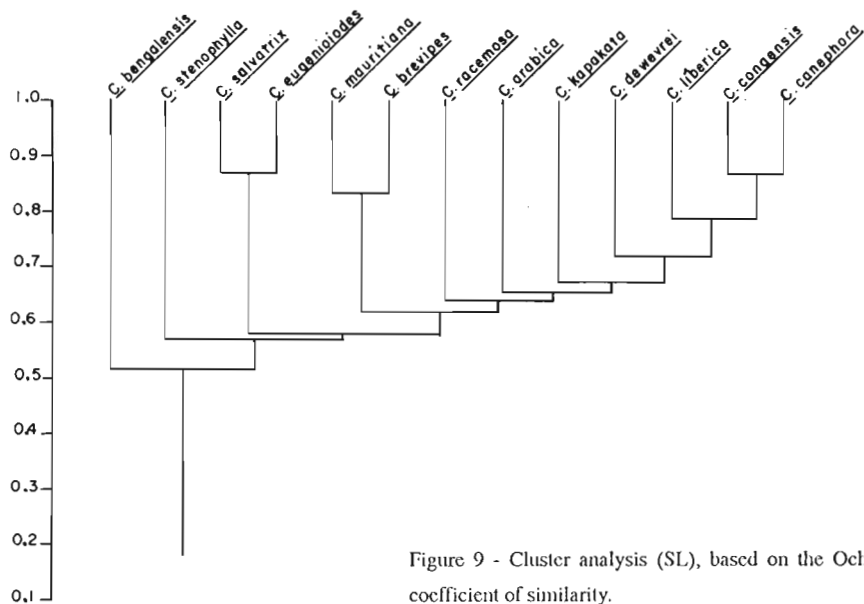


Figure 9 - Cluster analysis (SL), based on the Ochiai coefficient of similarity.

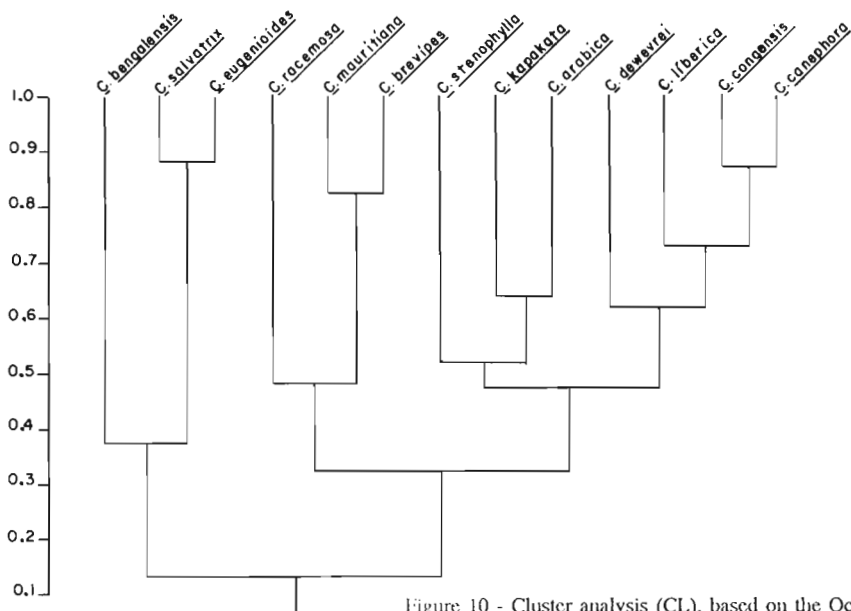


Figure 10 - Cluster analysis (CL), based on the Ochiai coefficient of similarity.

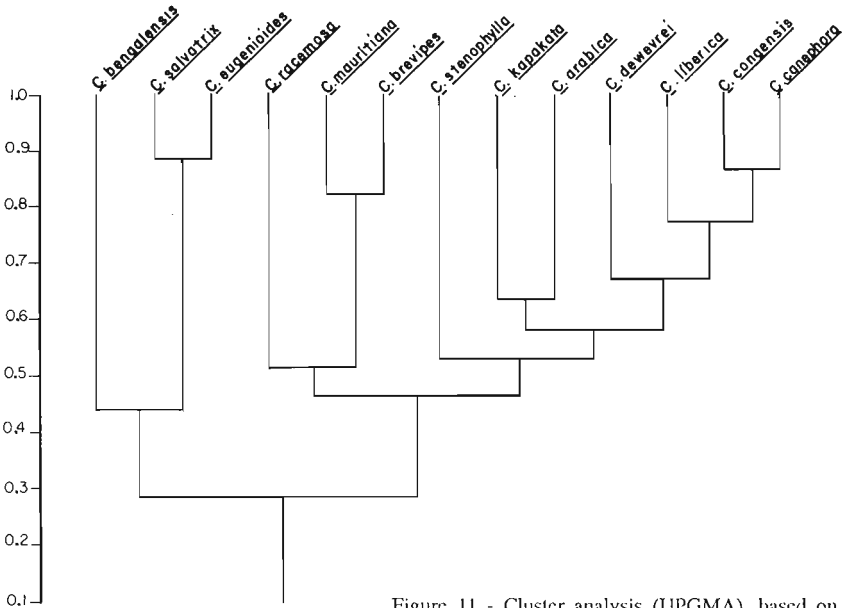


Figure 11 - Cluster analysis (UPGMA), based on the Ochiai coefficient of similarity.

participation of *C. eugenioides* in the formation of *C. arabica* is supported by all known data, including morphological similarities, chloroplast DNA, flavonoids and level of crossability (Carvalho and Monaco, 1967; Charrier, 1978; Lopes and Monaco, 1979; Berthou and Mathieu, 1982). It is known that *C. arabica* is distributed in restricted and isolated areas located in southwest Ethiopia, southeast Sudan and north of Kenya, where possibly its evolution took place after a duplication of an interspecific hybrid occurred (Carvalho and Monaco, 1967; Louarn, 1981; Berthou and Mathieu, 1982). This species can be considered a segmental allotetraploid, as defined by Stebbins (1950).

According to Charrier (1978), some types existing in the same diversification center in Central Africa suffered differentiation: to the west giving origin to *C. canephora*, *C. congensis*, *C. liberica*, *C. dewevrei*, *C. stenophylla*, etc.; to the south forming the species of the Mozambicoffea and Mascarocoffea subsections, and to the north giving origin to *C. arabica*. Since the diploid species share relations with only one genome of *C. arabica*, Charrier (1978) suggested that the other genome arose from an unknown species, which expressed fewer relationships with the genus *Coffea*.

Of the nine clusters presented, in five of them the species *C. stenophylla*, the only representative of the subsection Melanocoffea studied, showed greater similarity

with the species of the subsections *Erythrocoffea* and *Pachycoffea*, such as *C. canephora*, *C. congensis*, *C. dewevrei* e *C. liberica*, than to the species of the subsections *Mozambicoffea* and *Nanocoffea*.

As for the species *C. racemosa* of the subsection *Mozambicoffea*, its closest similarity was with the species *C. kapakata* of the same subsection (Carvalho and Monaco, 1967). There was no accordance between morphological and electrophoretic variability. The cross between them was also not successful (Carvalho *et al.*, 1985).

*C. bengalensis*, from Birmany, center and south India, is the most distinct of all the species studied. It belongs to the section *Paracoffea* according to Chevalier (1947) and to the genus *Paracoffea* according to Charrier (1978). It never formed specific similarity groups with the other studied species.

### ACKNOWLEDGMENTS

These investigations were supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). I wish to thank Dr. Alcides Carvalho for sample collection and Dr. George Shepherd for his help in the numerical analysis.

Publication supported by FAPESP.

### RESUMO

Os padrões isoenzimáticos para alfa e beta esterases, fosfatase alcalina, fosfatase ácida, desidrogenase málica e desidrogenase alcoólica foram determinadas em amostras de treze espécies do gênero *Coffea*. Os dados obtidos foram analisados através de três coeficientes de similaridade, Simple-Matching, Jaccard e Ochiai e por três diferentes métodos de agrupamento, o Single Linkage, Complete Linkage e Unweighted pair group using arithmetic averages.

As relações filogenéticas entre as doze espécies diplóides, e delas com a espécie tetraplóide *C. arabica*, mostraram que na maioria das vezes, houve maior similaridade entre espécies pertencentes a subseções diferentes do que entre aquelas de uma mesma subseção. Embora algumas espécies formem sempre grupos de maior similaridade, tanto através dos estudos de características morfológicas, de flavonóides, de cruzamentos interespecíficos, como aqueles de polimorfismo isoenzimático, o que se pôde verificar é que não há indicações de agrupamentos similares mais amplos, fundamentados nos estudos da maioria ou de todos esses diferentes processos de avaliação das interrelações filogenéticas e de afinidade.

### REFERENCES

- Allard, R.W., Kahler, A.L. and Weir, B.S. (1971). Isozyme polymorphism in barley populations. *Barley Genetics* 2: 1-13.

- Berthou, F. and Mathieu, C. (1982). Variation des organelles cellulaires dans les principales espèces de *Coffea*. Analyse des ADN chloroplastiques et mitochondriaux à l'aide des enzymes de restriction. *Colloque Scientifique International sur le café*. Salvador, Bahia, Brasil, 10: 421-431.
- Bridson, D.M. (1982). Studies in *Coffea* and *Psilanthus* (Rubiaceae subfam. Cinchonoideae) for part 2 of Flora of Tropical East África: Rubiaceae. *Kew. Bulletin* 36: 817-859.
- Brown, A.H.D. and Allard, R.W. (1970). Stimulation of the mating system in open pollinated maize populations using enzyme polymorphism. *Genetics* 66: 135-145.
- Buckley, D.P., O'Malley, D.M., Apsit, V., Prance, G.T. and Bawa, K.S. (1988). Genetics of Brazil nut (*Bertholletia excelsa*). 1. Genetic variation in natural populations. *Theor. Appl. Genet.* 76: 923-928.
- Carvalho, A. and Monaco, L.C. (1967). Genetic relationship of selected *Coffea* species. *Cienc. Cult.* 19: 151-165.
- Carvalho, A., Medicina Filho, H.P. and Fazuoli, L.C. (1985). Evolução e melhoramento do cafeeiro. *I Colóquio Internacional sobre Citogenética e Evolução de Plantas*, 215-234, Piracicaba, SP.
- Charrier, A. (1975). Contribution à l'étude génétique des Mascaro-coffea. 7<sup>e</sup> Colloque Scientifique International sur de café. Hambourg (9/14 juin 1975). ASI, 483-495.
- Charrier, A. (1978). La structure génétique des caféiers spontanés de la région Malgache (Mascaro-coffea). Leur relations avec les caféiers d'origine africaine (Eucoffea). *Memoires ORSTOM*, Paris, 97, 223 p.
- Chevalier, A. (1942). Les caféiers du globe. II. Iconographie des caféiers sauvages et cultivés et des Rubiacées prises pour des caféiers. In: *Encycl. Biol.* (Lechevalier, P., ed.) Paris, 36 p., 158 pl.
- Chevalier, A. (1947). Les caféiers du globe III. Systematique des caféiers et faux-caféiers, maladies et insects nuisibles. In: *Encycl. Biol.* XXVIII, Fas. III (Lachevalier, P., ed.), Paris, 356 pp.
- Hamrick, J.L., Linhart, Y.B. and Mitton, J.B. (1979). Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. *Ann. Rev. Eco. Syst.* 10: 173-200.
- Hamrick, J.L. and Loveless, M.D. (1986). Isozyme variation in tropical trees: procedures and preliminary results. *Biotropica* 18: 201-207.
- Leroy, J.F. (1980). Ecolution et taxogenese chez les Caféiers (*Coffea* L., *Psilantus* Hook & et *Nostolachna* Durand). Hypothese sur leur origine. *C.R. Acad. Sci. Paris* 291: 593-596.
- Leroy, J.F. (1982). L'Origine Kenya du genre *Coffea* L. et la radiation des espèces à Madagascar. *Colloque Scientifique International sur le Café*, Salvador, Bahia, Brasil 40: 413-442.
- Lewontin, R.C. (1984). Detecting population quantitative characters as opposed to gene frequencies. *Ann. Nat.* 123: 115-124.
- Lopes, C.R. and Monaco, L.C. (1979). Chemotaxonomic studies of some species of the genus *Coffea*. *J. Plantat. Crops* 7: 6-14.
- Lopes, C.R. and Shepherd, G.P. (1991). Phylogenetic studies of some species of the genus *Coffea* - I - Numerical analysis of flavonoid compounds. *Rev. Bras. Genet.* 14: 425-435.
- Louam, J. (1982). Bilan des hybridations interspecifics entre caféiers africains diploides en collection au Côte d'Ivoire. *Colloque Scientifique International sur le Café*. Salvador, Bahia, Brasil, 10: 375-383.
- Rohlf, J.F. (1988). *NTSYS-pc. Numerical taxonomy and multivariate analysis system.* (Exeter Publishing, Ltd, ed.), New York, 150 pp.

- Scandalios, J.G. and Sorenson, J.C. (1977). Isozymes in plant tissue culture. In: *Applied and fundamental aspects of plant cell, tissue, and organ culture* (Reinert, J. and Bajaj, P.S., eds.). Berlin, Springer Verlag, pp. 719-789.
- Sneath, P.H.A. and Sokal, R.R. (1973). *Numerical taxonomy. The principles and practice of numerical classification*. San Francisco W.H. Freeman, 573 pp.
- Stebbins, G.L. (1950). *Variation and evolution in plants* (Columbia Univ.) Columbia Univ. Press, New York, 643 pp.
- Steiner, W.W.M. and Joslym, D.J. (1979). Electrophoretic techniques for the genetic study of mosquitoes. *Mosquito News* 39: 35-54.
- Turner, J.R.G., Johnso, M.S. and Eanes, W.F. (1979). Contrasted modes of evolution in the same genome: allozymes and adaptive change in *Heliconius*. *Proc. Natl. Acad. Sci.* 76: 1924-1928.
- Wolff, K. (1991). Genetic analysis of morphological variability in three *Plantago* species with different mating systems. *Theor. Appl. Genet.* 81: 111-118.
- Wrigley, G. (1988). Botany. In: *Coffea*, AICTA. Longman, Scientific and Technical eds. (London). John Wiley & Sons (New York), pp. 61-108.

(Received November 27, 1991)